4374

Cyclic Nucleotide-Independent Protein Kinases from Rabbit Reticulocytes

Site-Specific Phosphorylation of Casein Variants

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Purified variants of α_{s1} casein and β casein have been used to examine the substrate-specificity of two adenosine-3′:5′-monophosphate-independent protein kinase activities purified from rabbit reticulocytes. These enzymes, identified as casein kinase I and II were stimulated by monovalent cations. Casein kinase I had a pH optimum between 6.8 and 8.0, used ATP as the phosphate donor and preferentially phosphorylated serine. Casein kinase II had optimal activity at pH 8.5 to 10, utilized both ATP and GTP in the phosphotransferase reaction and modified predominantly threonine residues. The chymotryptic peptides of the phosphorylated casein variants were analyzed by thin-layer electrophoresis and ascending chromatography and the phosphorylated peptides were identified by autoradiography. In the β caseins, the phosphorylated peptides observed with casein kinase I were different from those obtained with casein kinase II. With the α_{s1} caseins, the same phosphorylated peptide was observed with both protein kinases. The phosphorylation sites in the α_{s1} and β caseins have been assigned on the basis of the amino acid analyses of the phosphorylated chymotryptic peptides and the phosphoserine and phosphothreonine determinations in conjunction with the known primary sequences. Casein kinase I phosphorylated Ser-22 in β-A^2 casein and Ser-41 in α_{s1}-A casein, suggesting that this protein kinase recognized the primary sequence Glu-X-Ser. Thr-41 in β-A^2 casein and Thr-49 in α_{s1}-A casein were the primary sites phosphorylated by casein kinase II. The recognition determinants for casein kinase II appeared to be Thr-Glu-Asp. When endogenous phosphate was removed from the caseins, the rate of phosphorylation of β casein was diminished by greater than six-fold indicating that negative charge near the phosphorylation site facilitated phosphorylation.

Two cAMP-independent protein kinases which modify casein have been highly purified from rabbit reticulocytes by ion-exchange chromatography as previously described by Hathaway and Traugh [1]. These enzymes have been designated casein kinase I and casein kinase II in order of elution from DEAE-cellulose. Casein kinase I is a single polypeptide chain of molecular weight 37000 and preferentially uses ATP. Casein kinase II is a tetramer (M_r 130000) and utilizes both ATP and GTP. Enzymes similar to casein kinase II have also been highly purified from rat liver [2] and Novikoff ascites tumor cells [3].

Little is known about the mechanism of action or the biological role of the cAMP-independent protein kinases from reticulocytes, although several translational initiation factors have been identified as endogenous substrates [4–9]. Initiation factors eIF-4B and eIF-5 have been shown to be phosphorylated by casein kinase I while initiation factors eIF-2, eIF-3, eIF-4B and eIF-5 are modified by casein kinase II [4]. In these studies the purified casein variants have been used to examine the substrate specificity of protein kinases since the caseins have well-established primary structures [10–15]. Casein occurs as a mixture of phosphoproteins arbitrarily designated as α_{s1} (50%), β (33%), $\varkappa$ (15%) and γ (3%) caseins [16,17]. Each of these caseins has a unique primary structure except for the γ caseins, which are large fragments of β casein (for review see [17]). Several polymorphic forms are known for each of the classes of casein. These variants have slightly different electrophoretic mobilities and vary from each other by one or several amino acids. We have previously shown that different sites in β casein B are phosphorylated by casein kinase I, casein kinase II and the cAMP-dependent protein kinases using a two-dimensional fingerprinting procedure [18]. This article is an extension of that work, and the

Abbreviation. Cyclic AMP or cAMP, adenosine 3′:5′-monophosphate.

Enzymes. Chymotrypsin (EC 3.4.21.1); acid phosphatase (EC 3.1.3.2).

phosphorylation sites in the α_{s1} and β caseins are identified.

EXPERIMENTAL PROCEDURE

Materials

[γ-^{32}P]ATP and [γ-^{32}P]GTP were prepared as previously described [4] by a modification of the procedure of Glynn and Chappell [19]. Chymotrypsin-A_4 was obtained from Boehringer-Mannheim and ninhydrin spray from J. T. Baker Chemical Co. Precoated plastic-backed thin-layer cellulose sheets (20 × 20 cm, 0.16-mm thick, without fluorescent indicator) were purchased from Eastman. The protein kinases were isolated and purified by chromatography on DEAE-cellulose, phosphocellulose and sulfopropyl-Sephadex as previously described [1], and were generously provided by Dr Gary M. Hathaway.

Casein Variants

The variants of α_{s1} and β caseins were highly purified from the milk of cows homozygous for a particular variant. The α_{s1} casein variants (A, B, and C) and the β caseins (A^1, A^2, A^3 and B) were used in these studies. The primary sequence for each of these variants has been determined. Glu-14 to Ala-26 present in α_{s1}-B casein was deleted in α_{s1}-A casein [10,15] while Glu-192 was replaced with Gly in α_{s1}-C casein [10]. In the β-caseins, using the A^2 variant as standard, the following alterations were identified: β casein A^1, Pro-67/His [11]; β casein A^3, His-106/Gln [12]; β casein B, Pro-67/His, Ser-122/Arg [11]. Dephosphorylated α_{s1}-B and β-A^2 caseins were obtained by incubation of the variants with acid phosphatase as described by Bingham et al. [20]. The phosphorus content of the native and dephosphorylated casein variants were determined by the method of Meun and Smith [21]. The α and β casein variants used in these studies contained the theoretical number of phosphorus atoms expected for the fully phosphorylated native form [10–15]. Less than 3% of the phosphorus remained after dephosphorylation. The purity and extent of phosphorylation of the casein variants were also verified by polyacrylamide gel electrophoresis as described [20,22]. Commercial casein from Matheson, Coleman and Bell was dephosphorylated as described by Reimann et al. [23]. Concentrations of casein were determined by the method of Lowry et al. [24] with bovine serum albumin as standard.

Phosphorylation of Casein

Casein was phosphorylated in a standard reaction mixture of 0.07 ml which contained the following: 50 mM Tris-HCl, pH 7.0; 10 mM $MgCl_2$; 140 mM KCl; 0.14 mM [γ-^{32}P]ATP or [γ-^{32}P]GTP (specific activity about 300–500 dis. × min^{-1} × $pmol^{-1}$); 2 mg of casein/ml; and 0.4 to 1.5 U of either casein kinase I or II. A unit of protein kinase activity has been defined previously [18] as the amount of enzyme which catalyzes the incorporation of 1 pmol phosphate/min into dephosphorylated commercial casein at 30 °C. Reactions were initiated by addition of ATP or GTP and incubated for 15 min at 30 °C. Under these conditions less than 5% of substrate was reacted. The reaction was terminated by spotting onto filter paper squares and the protein was precipitated in 10% trichloroacetic acid. The samples were washed and prepared for scintillation counting as previously described [4].

Maximal phosphorylation of casein was obtained as described above except that more protein kinase was added, and the incubation period was increased to 8 to 12 h. In a typical experiment 6 to 10 U and 29 nmol of radioactive ATP were used to phosphorylate casein at a concentration of 2 mg/ml in a 0.21 ml reaction mixture. After 4 h, more protein kinase (7 U) and ATP (15 nmol) were added in a small volume.

Phosphoserine and Phosphothreonine Determination

Casein was phosphorylated with either casein kinase I or II, and the reaction was terminated by the addition of 100% cold trichloroacetic acid to a final concentration of 25%. The precipitate was collected by centrifugation at 10000 × *g* for 10 min, washed two times with the same volume of 5% trichloroacetic acid, and once with ethanol/ether (2/1) and ether. After dissolving the precipitate in 0.2 M NH_4HCO_3, pH 8.6, the casein was diluted to 1 to 2 mg/ml and immediately dialyzed against water at 4 °C. Hydrolysis was carried out in 4 M HCl in a sealed evacuated tube at 110 °C for 4 h. The hydrolysate was lyophilized and the residue taken up in a small volume of water. Phosphoserine and phosphothreonine residues were analyzed by thin-layer electrophoresis in 1.8% formic acid/7.3% acetic acid pH 1.9 at 500 V for 2 h. Phosphoserine and phosphothreonine were used as internal standards and visualized by staining with ninhydrin. The radioactivity labeled residues were identified by autoradiography with Kodak No screen X-ray film. The spots corresponding to phosphoserine, phosphothreonine, P_i and partially hydrolyzed starting material were scraped from the thin-layer chromatography plate and counted in vials containing toluene scintillation cocktail.

Peptide Mapping

Peptide maps of the chymotryptic digests of maximally phosphorylated casein were obtained using thin-layer electrophoresis in the first dimension and ascending chromatography in the second dimension as previously described [18]. Peptides were located

by spraying with ninhydrin and the radioactivity was identified by autoradiography.

Amino Acid Analysis of ^{32}P-Labeled Chymotryptic Peptides

The radioactive peptides were scraped from two plates and extracted successively with 1 ml each of 0.5 M acetic acid, water, and 0.5 M pyridine. The combined supernatants were evaporated to dryness in a 25 ml hydrolysis tube and the residue was hydrolyzed *in vacuo* in 6 M HCl (1 ml) at 110 °C for 24 h. Amino acid analysis was performed in a Beckman amino acid analyzer (model 120C) equipped with a 20-mm light path cuvette and expanded recorder range card which allowed measurement of a few nanomoles of sample.

RESULTS

Characterization of the Phosphorylation Reaction

Phosphorylation of α_{s1}-C casein, β-A^2 casein and dephosphorylated commercial casein was examined with respect to monovalent and divalent cation concentration, pH optimum, and phosphate donor using casein kinase I and II. The results of these studies are summarized in Table 1. The optimum concentrations for KCl were found to be essentially the same using the three different substrates and ranged from 140 to 300 mM for casein kinase I and 100 to 250 mM for casein kinase II. KCl was shown to stimulate both protein kinase activities approximately two-fold (data not shown). The optimum concentrations for magnesium ions were found to be essentially the same for both enzymes using the three different substrates and ranged from 2 to 7 mM. At higher cation concentrations the rate decreased except for the phosphorylation of commercial casein with casein kinase II; the rate for the latter was unaffected between 5 and 15 mM $MgCl_2$. An absolute requirement for magnesium chloride in the phosphorylation reactions was observed with both enzymes. Casein kinase I showed a broad pH optimum between 6.8 and 8.0, with a 20% decrease in activity at pH 6.0 and 8.5. Casein kinase II was optimally active at pH 8.5 to 10.0; this activity was decreased 2.5-fold at pH 7.0.

As shown previously with commercial casein [1], casein kinase II utilized both ATP and GTP as the phosphate donor, with a preference for ATP. Casein kinase I had a higher specificity for ATP and at nucleotide concentrations of 0.14 mM the reaction was 15 times greater with ATP than with GTP. The nucleotide specificity of casein kinase I and II was also observed with α_{s1}-C and β-A^2 caseins and reflected differences in the K_m values for ATP and GTP with the two enzymes.

Phosphorylation of the α_{s1} and β Casein Variants

The rates of phosphorylation of the casein variants with casein kinase I and II were examined. The relative rates for the phosphorylation of the α_{s1} variants with casein kinase I ranged from 0.4 to 1.2 (Table 2). Phosphorylation of the β caseins was approximately two times greater than that of the α_{s1} caseins; little difference in rate was observed between the individual β variants. A comparison of the rates of phosphorylation of the α_{s1} caseins with casein kinase II revealed that the A and C variants were 9–10 times more reactive than the B variant. With casein kinase II, the β caseins were phosphorylated 18–25 times faster than α_{s1} casein B, and 2–3 times greater than α_{s1}-A or α_{s1}-C.

When enzymatically dephosphorylated α_{s1}-B was used as substrate, a 30% decrease in the rate of phosphate transfer with casein kinase I was observed. With dephosphorylated β-A^2 casein as substrate, the rate of phosphorylation was decreased dramatically

Table 1. *Phosphorylation of casein by casein kinase I and II*
The rate of ^{32}P incorporation was measured using the standard assay conditions described in Experimental Procedure except that the parameters indicated were varied. Buffers used were 50 mM 2-(*N*-morpholino)ethanesulfonic acid for pH 6.0–6.8; 50 mM Tris-HCl for pH 7.0–8.5; 50 mM sodium glycinate for pH 9–10. Values for ATP/GTP are the ratio of initial rates using 0.14 mM ATP or GTP; values for commercial casein were obtained from [1]; n.d., not determined

Casein kinase	Casein	Optimum conditions			^{32}P incorporated ATP/GTP
		KCl	$MgCl_2$	pH	
		mM			
I	α_{s1}-C	140–300	2– 5	n.d.	12.2
	β-A^2	140–240	2– 7	6.8– 8.0	10.0
	Commercial (dephosphorylated)	140–300	2– 5	6.8– 8.0	15.0
II	α_{s1}-C	n.d.	3– 7	n.d.	3.0
	β-A^2	100–250	3– 7	8.5–10.0	2.9
	Commercial (dephosphorylated)	100–220	5–15	8.5–10.0	3.0

Table 2. *Relative rates of phosphorylation of casein variants*
The rate of phosphorylation was determined using the standard assay conditions described under Experimental Procedure

Casein variant	Casein kinase I		Casein kinase II	
	^{32}P incorporated	relative rate	^{32}P incorporated	relative rate
	nmol × min^{-1} × ml^{-1}		nmol × min^{-1} × ml^{-1}	
α_{s1}-A	3.36	1.2	9.83	0.9
α_{s1}-B	1.58	0.6	1.43	0.1
α_{s1}-B (dephosphorylated)	1.16	0.4	1.05	0.1
α_{s1}-C	2.78	1.0	11.06	1.0
β-A^1	4.58	1.6	19.89	1.8
β-A^2	4.53	1.6	28.00	2.5
β-A^2 (dephosphorylated)	0.27	0.1	4.74	0.4
β-A^3	5.04	1.8	20.99	1.9
β-B	4.69	1.7	19.40	1.8

Table 3. *Phosphoserine and phosphothreonine determinations*
Determinations were done as described in the text under Experimental Procedure

Casein kinase	Casein	^{32}P incorporated		Ratio phosphoserine/ phosphothreonine
		phosphoserine	phosphothreonine	
		dis./min		
I	α_{s1}-C	768	20	38
	β-A^2	338	25	14
II	α_{s1}-C	444	1014	0.43
	β-A^2	48	699	0.07

by six–ten-fold with casein kinase I and II. This indicates that the endogenous phosphoryl groups on β-casein enhanced phosphorylation by both enzymes.

The apparent K_m values for casein kinase I and II with several α_{s1} and β casein variants were determined. Initial rates were obtained with casein concentrations up to 5 mg/ml and the kinetic data were fitted to the Michaelis-Menten equation using a weighted least squares analysis. Casein kinase I had K_m values ranging from 0.4 to 0.6 mg/ml and values of 0.8 to 1.7 were obtained with casein kinase II. The K_m values obtained with whole casein (α_{s1}-B, β-A^1, $\varkappa$-A) were comparable to those observed with the variants; 0.6 for casein kinase I and 1.1 for casein kinase II.

The extent of phosphate incorporation into the casein variants was determined using high concentrations of enzyme. Greater than one mole of phosphate was incorporated per mole of β-A^2 or β-B casein with casein kinase II. In similar studies using casein kinase I, one mole of phosphate was incorporated per mole of either α_{s1} casein C or the A^2 and B variants of β-casein.

Phosphoserine and Phosphothreonine Determinations

Casein variants, α_{s1}-C and β-A^2, were maximally phosphorylated with [γ-^{32}P]ATP using either casein kinase I or II and subjected to partial acid hydrolysis. The hydrolysates were analyzed for phosphoserine and phosphothreonine by high-voltage electrophoresis. The majority of the radioactivity migrated with the phosphoserine and phosphothreonine standards; however, a portion of the radioactivity migrated with inorganic phosphate and with partially hydrolyzed casein. As shown in Table 3, casein kinase I preferentially phosphorylated serine residues. Casein kinase II preferentially modified threonine, but serine was also significantly phosphorylated in α_{s1}-C casein. Thus casein kinase I preferentially modified serine, while casein kinase II phosphorylated threonyl residues.

Identification of Phosphorylation Sites

In order to identify the phosphorylated residues, α_{s1}-A and β-A^2 caseins were maximally phosphorylated with either casein kinase I and ATP, or casein kinase II and ATP or GTP, and subjected to chymotryptic digestion. Maps of the chymotryptic peptides were obtained by thin-layer electrophoresis followed by ascending chromatography. Autoradiograms of the peptide maps for the two casein variants are shown in Fig. 1. Different phosphorylated peptides were obtained from β-A^2 casein when casein kinase I or

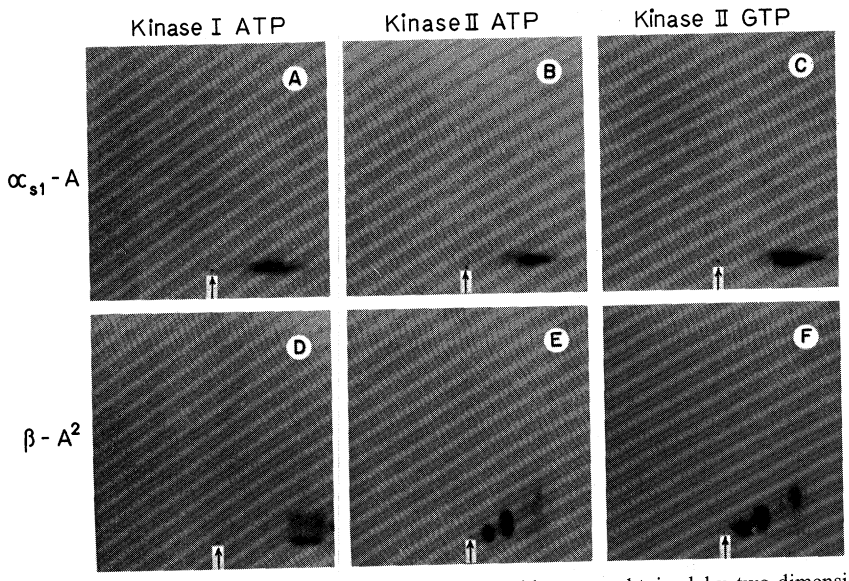

Fig. 1. *Separation of phosphorylated peptides.* Autoradiography of the peptide maps obtained by two-dimensional fingerprinting of the chymotryptic digests of α_{s1} casein A phosphorylated by (A) casein kinase I with ATP; (B) casein kinase II with ATP; (C) casein kinase II with GTP; and of β casein A^2 phosphorylated by (D) casein kinase I with ATP; (E) casein kinase II with ATP; (F) casein kinase II with GTP

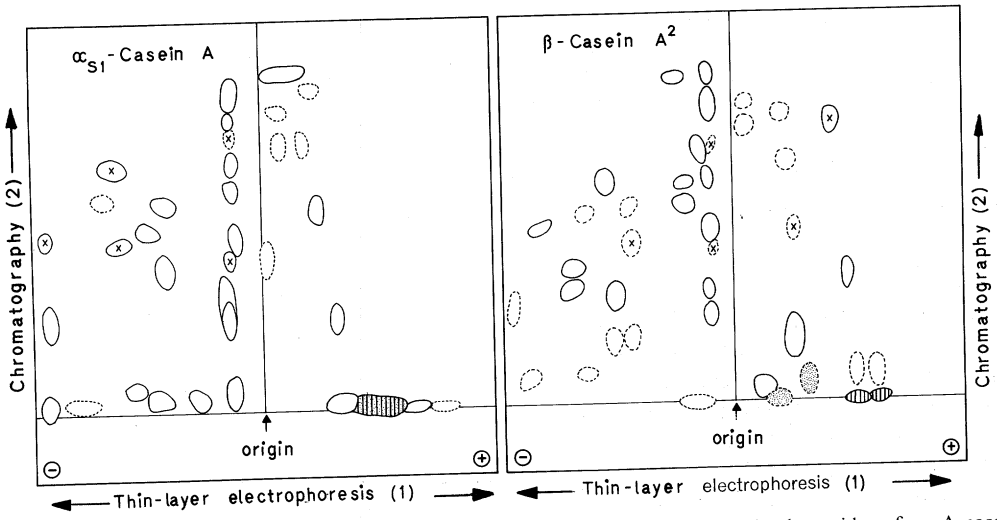

Fig. 2. *Diagram of the chymotryptic peptides from β casein A^2 and α_{s1} casein A.* The ninhydrin-stained peptides of α_{s1}-A casein are on the left, and β-A^2 casein on the right. Solid lines indicate heavily stained peptides; dashed lines indicate lightly stained peptides; X denotes color variation of brown and grey. Sites phosphorylated by casein kinase I are shaded with lines, and those phosphorylated by casein kinase II are shaded with dots

casein kinase II was used to phosphorylate the variant. Identical results were obtained with casein kinase II using ATP or GTP. The substrate specificity of each enzyme was substantiated further when identical results were obtained with the β-variants A^1, A^3, (data not shown) and B [18]. When α_{s1}-A casein was phosphorylated by casein kinase I or II, the radioactive phosphate was found in one elongated spot. As shown by the peptide maps for α_{s1}-A and β-A^2 caseins (Fig. 2), the casein kinases modified different sites in the β-variants, but the phosphate acceptor sites for these enzymes were in the same chymotryptic peptide in the α_{s1} variants.

The amino acid compositions of the phosphorylated peptides from β-A^2 and α_{s1}-A caseins were determined and compared with the amino acid composition of the casein variants. The two radioactive peptides obtained after chymotryptic digestion of β-A^2 casein phosphorylated with casein kinase I were designated 1 and 2 in order of increasing electrophoretic mobility. They were found to have similar amino acid compositions and corresponded to the peptide fragments, Arg-1 to Lys-28 and Glu-4 to Asn-27 (Table 4). It should be noted that these peptides resulted from cleavage at secondary sites by chymotrypsin. Cleavage at the carboxyl group of threonine and lysine have

been reported to occur with prolonged digestion periods and high ratios of enzyme to substrate, especially when basic residues adjacent to these less susceptible bonds are present [25,26]. The cleavage site at Lys-28 has been shown to occur *in vivo* giving rise to the γ^1 caseins [17]. The two peptides of β-A^2 casein phosphorylated by casein kinase II were also similar in composition and corresponded to Lys-29 to Phe 52 and Lys-29 to Leu-58. These were primary cleavage sites. The amino acid composition of the phosphorylated peptide obtained from α_{s1}-A casein was consistent with that of the peptide Val-37 to Gln-79 (Table 4). As in the case with some of the peptides from β casein, this peptide was the result of cleavage at secondary sites.

DISCUSSION

The two cAMP-independent protein kinases used in these studies were shown to be distinctly different enzymes. The activities were stimulated by monovalent cations but differed with respect to pH optima, phosphoryl donor and substrate specificity. Casein kinase II resembled the casein kinases isolated from rabbit erythrocytes by Kumar and Tao [27] which were optimally active at pH 9 and utilized both ATP and GTP. Casein kinases from yeast [28], the Golgi apparatus of rat mammary glands [29] and rat liver cytosol [30] have been reported to have pH optima of 6.8 to 7.5 as was found for casein kinase I. The K_m values were similar to those reported for casein kinases from other sources [27,29].

The substrate specificity of casein kinase I and II was well defined with the β variants. Two of the chymotryptic peptides were radioactive after the phosphorylation of β-A^2 casein by casein kinase I. These were quite different from the two radioactive peptides obtained after phosphorylation with casein kinase II.

On the basis of the amino acid analyses of the phosphorylated peptides and the known primary sequences of the casein variants, the specific residues in β-A^2 casein phosphorylated by each kinase were assigned (Fig. 3). With casein kinase I, two overlapping peptides were phosphorylated, Arg-1 to Lys-28 and Glu-4 to Asn-27. The only possible phosphorylation sites in these peptides were Ser-22 and Thr-24. Since radioactive phosphoserine was detected in the partial acid hydrolysates (Table 3), it follows that only Ser-22 was modified. In the two overlapping peptides obtained after phosphorylation by casein kinase II, (Lys-29 to Leu-58 and Lys-29 to Phe-52), the only potential site for phosphorylation in both peptides was Thr-41. In one of the peptides Thr 55 could have been modified also; however, it is unlikely since a third phosphorylated peptide, Ala-53 to Leu-58 would have been detected. Thus, casein kinase II modified Thr-41 in β casein A^2.

The same phosphorylated peptide was obtained from α_{s1}-A casein after modification by either casein

Table 4. *Amino acid composition of the phosphorylated chymotryptic peptides from β casein A^2 and α_{s1} casein A*
Amino acid analyses were performed as described under Experimental Procedure and the data presented as moles of amino acid per mole of peptide. The values in parentheses are considered to be impurities. A dash indicates a value of less than 0.1 and n.d. indicates that no determination was made. Methionine was observed as the sulfoxide

Amino acid	Casein kinase I; β-A^2 casein				Casein kinase II; β_{s1}-A^2 casein				Casein kinase I; α_{s1}-A casein	
	phosphopeptide 1		phosphopeptide 2		phosphopeptide 1		phosphopeptide 2		phosphopeptide	
	observed	expected Arg^1-Lys^{28}	observed	expected Glu^4-Asn^{27}	observed	expected Lys^{29}-Leu^{58}	observed	expected Lys^{29}-Phe^{52}	observed	expected Val^{37}-Gln^{78}
Lysine	0.8	1	(0.3)	–	3.3	3	2.6	3	1.4	2
Histidine	–	–	–	–	1.0	1	0.8	1	(0.6)	–
Arginine	1.4	2	1.1	1	–	–	–	–	–	–
Aspartate	2.2	2	1.8	2	3.1	2	2.3	2	4.7	5
Threonine	1.1	1	1.1	1	1.5	2	0.7	1	1.6	1
Serine	3.7	5	3.4	5	2.1	2	1.9	1	6.5	8
Glutamate	7.0	7	6.0	6	12.0	12	10.0	10	12.0	12
Proline	n.d.	1	n.d.	1	n.d.	1	n.d.	1	1.1	1
Glycine	1.6	1	1.8	1	1.0	–	1.4	–	1.8	1
Alanine	(0.4)	–	(0.2)	–	0.8	1	0.7	–	2.0	2
Valine	1.8	2	1.7	2	(0.6)	–	(0.5)	–	2.9	3
Methionine	–	–	–	–	–	–	–	–	1.6	2
Isoleucine	2.9	3	2.6	3	2.1	2	1.6	2	3.9	4
Leucine	2.3	3	2.1	2	1.4	2	1.2	1	0.8	1
Tyrosine	–	–	–	–	–	–	–	–	–	–
Phenylalanine	–	–	–	–	2.3	2	1.6	2	–	–
Tryptophan	n.d.	–	n.d.	–	n.d.	–	n.d.	–	n.d.	–

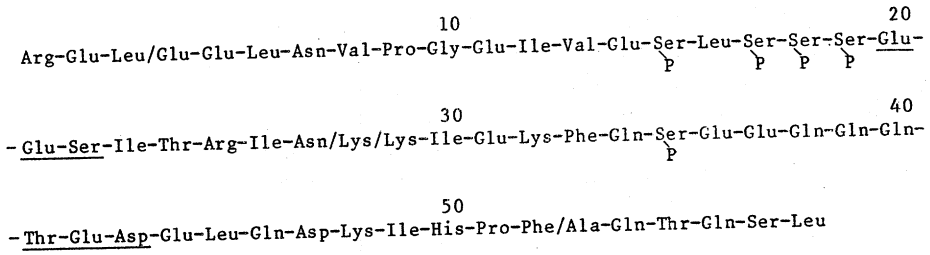

Fig. 3. *Identification of phosphorylation sites in β casein A*[2]

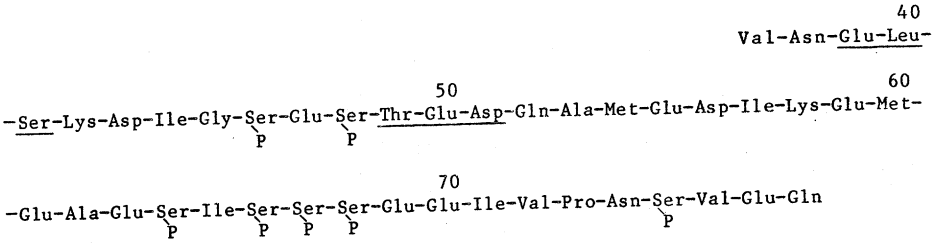

Fig. 4. *Identification of phosphorylation sites in α_{s1} casein A*

kinase I or II. Two potential phosphorylation sites Ser-41 and Thr-49, were available in the peptide Val-37 to Gln-78. Since a serine residue was phosphorylated by casein kinase I, Ser-41 was the site of phosphorylation (Fig. 4). Evidence has been obtained recently for the phosphorylation of Ser-41 *in vivo* [31]. Threonine was the major phosphorylated residue observed in the partial acid hydrolysates after modification by casein kinase II. Thus the major site of phosphorylation by casein kinase II was Thr-49. Since a significant amount of phosphoserine was also observed with casein kinase II, Ser-41 appeared to be a secondary phosphorylation site under conditions of prolonged incubation.

Of the 20 potential phosphorylation sites in β-A^2 casein (11 serine and 9 threonine residues) and the 13 potential sites in α_{s1}-A casein (8 serine and 5 threonine residues) only one site in each variant was phosphorylated by the individual kinases. Thus it appeared that the protein kinases have different recognition determinants. The primary sequence recognized by casein kinase I appeared to be Glu-X-Ser, with X being any amino acid. Except for Ser-22, the proposed site of phosphorylation in β-A^2, no other serine or threonine residue had this feature. However, in the α_{s1} caseins, there was a potential site at Ser-191. The hydrophobic nature of the C-terminal end of the molecule could be a factor in preventing phosphorylation of this serine residue. With casein kinase I, the β caseins were found to be 2–3 times more reactive than the α_{s1} caseins. This suggests that other amino acids in the vicinity of the phosphorylation site exerted some effect on the rate of phosphorylation. It is possible that the increased rate of phosphorylation with the β caseins was due to the negatively charged region on the N-terminal side of Ser-22 (Fig. 3). Kemp et al. [32,33] have shown that the rate of phosphorylation by the cAMP-dependent protein kinases was affected by substitution of amino acids around the phosphorylation site of the substrate.

The recognition determinant for casein kinase II appeared to be a Thr-Glu-Asp. Analysis of the primary structures indicated that α_{s1} and β casein had only one residue with this characteristic, Thr-41 in the sequence Thr-Glu-Asp-Glu and Thr-49 in the sequence Thr-Glu-Asp-Gln respectively. Since phosphoserine was also found in the partial acid hydrolysates of α_{s1}-C casein phosphorylated by casein kinase II, it suggests that Ser-41 was also modified. It is possible that the Asp one residue away on the C-terminal side of serine or threonine is a sufficient determinant for casein kinase II. This would make Ser-41 in Ser-Lys-Asp a minor site of phosphorylation.

It should be noted that the casein kinases studied are quite distinct from the cAMP-dependent protein kinases, which require basic residues on the N-terminal side of the phosphorylation site [32–34], and the casein kinase associated with the Golgi apparatus from mammary gland. The latter enzyme which is responsible for the endogeneous phosphoserine residues in whole casein required a phosphoseryl or glutamyl residue on the C-terminal side of the phosphorylation site [11].

When dephosphorylated β casein was used as substrate, a dramatic decrease in the rate of phosphorylation with both casein kinase I and II was observed. A decreased rate of phosphorylation had similarly been observed with a yeast casein kinase using dephosphorylated α_{s1} and β caseins [28]. These results support the idea that the presence of the

phosphoryl residues near the serine or threonine residue facilitates phosphorylation as has been suggested for the casein kinase activities from the Golgi apparatus of mammary gland [11].

We are grateful to Dr Gary M. Hathaway for the purified protein kinases and to Dr Richard Palmieri for performing the amino acid analyses and to both of them for many helpful discussions. We thank Merton C. Groves for supplying some of the purified casein variants and Rae Greenberg for supplying unpublished tryptic peptide maps of several variants. This research was supported by Grant GM 21424 from the United States Public Health Service.

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